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IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF	:
TETSUYA SUGA, ET AL	: EXAMINER: BROOKS, KRISTIE LATRICE.
SERIAL NO: 10/692,684	:
FILED: OCTOBER 27, 2003	: GROUP ART UNIT: 1616
FOR: IMMUNE ACTIVATOR:	

DECLARATION UNDER 37 C.F.R. §1.132

COMMISSIONER FOR PATENTS
P.O. BOX 1450
ALEXANDRIA, VA 22313

SIR:

Now comes Yasuyo Suga, who declares and states that:

1. I am a graduate of University of Kyoto Institute of Technology, and received my bachelor degree in the field of agriculture, in the year 1991;

2. I have been employed by Ajinomoto Co., Inc., for 19 years as a researcher in R&D department involving in the study of medicine;

3. I am a named inventor of the above-identified application.

4. I have read and understand the present application and the Office Actions to date, including the references cited therein;

5. It is my opinion that the skilled artisan would not modify a disclosure of β-glucans from mushrooms based on a disclosure of β-glucans from yeast. This opinion is based on the fact that there are various types of β-glucans, and there are differences in structure between β-glucans from mushrooms and β-glucans from yeast.

Specifically, β-1,3-glucans derived from mushroom, in general, comprises β-1,6-glucans. On the other hand, β-1,3-glucans derived from yeast, does not comprise β-1,6-glucans. Thus, the β-glucans derived from mushroom is distinct from β-glucans derived from yeast as to the presence of β-1,6-glucans.

Support for the foregoing is provided by the following references, which were submitted on June 25, 2008:

a. Documents regarding Lentinula edodes (Shiitake) (Lentinan)

Sasaki T., Takasuka N., Carbohydr. Res., 47, 99 (1976)

Sasaki T., Takasuka N., Chihara G., Maeda Y. Y., Gann, 67, 191 (1976)

Saito H., Ohki T., Sasaki T., Biochem. 16, 908 (1977)

b. Document regarding Schizophyllum commune (Sizofiran)

Tabata K., Ito W., Kojima T. et al Carbohydr. Res., 89, 121 (1981)

c. Document regarding Selerotium (Seleroglucan)

Falch B H, Espevik T, Ryan Let al. Carbohydr. Res., 329, 587 (2000)

In addition to the foregoing evidence that establishes that mushrooms comprise β-1,6-glucans the following references were filed with the response on January 29, 2009 to show that yeast disclosed by Kropf et al are devoid of β-1,6-glucans in addition to aforementioned references that show that the only β-glucans derived from yeast are β-1,3-glucans or β-glucans having both 1→3-linked and 1→6-linked glucose residues, which are distinct from the β-1,6-glucans derived from mushrooms:

a. "Zymosan", Wikipedia entry retrieved January 26, 2009 at http://en.wikipedia.org/wiki/Zymosan;

b. "β-glucan", Wikipedia entry retrieved January 27, 2009 at http://en.wikipedia.org/wiki/Beta-glucan;

c. Tada R, et al., Glycoconj. J. 25:851-861, 2008;

d. Oshiman K, et al., Planta Med. 8:610-614, 2002.

References (a) - (c) show that β-glucans derived from yeast are mostly β-glucans having β-1,3-linked main chains (partly, having β-1,6-linked branched chains (residues)). Reference (d) shows that β-glucans derived from mushroom has β-1,6-linked main chains, not β-1,6-linked as a residue.

6. With respect to the particle size of β-glucans "wherein the superfine particles have an average particle diameter of 10 μm or less" there is no direct relation between absorbability and the medical effect as supported by the following references are attached to this Declaration:

a. Suga et al., Biotherapy. 17(3):267-273, 2003;

b. Suga et al., Biotherapy. 19(3):273-278, 2005;

c. Shen et al., Biomedial Research. 28(2):71-77, 2007.

Moreover, when finding the ingredient of an orally effective pharmaceutical, for example, even if absorbability is improved to some extent, degradation is apt to occur when the absorbability is enhanced. Even if the degradation does not occur, pharmacological effect is not necessarily produced, and many difficult problems may newly arise. Therefore, even if the particle size becomes smaller, both of absorbability and immune activating effect are not necessarily improved. Thus, the effect of the present invention as demonstrated in the my Declaration under 37 C.F.R. §1.132 executed on September 7, 2009, is unexpected.

7. I declare further that all statements made of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

8. Further Declarant saith not.

Yasuyo Suga
Yasuyo Suga

September 22, 2010
Date

D1

原　著

微粒子化キノコエキスのマウス担癌モデルにおける抗腫瘍効果

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要旨　β-1,3-グルカン（レンチナン）のもつ抗腫瘍効果は，経口投与では認められない。今回われわれは，溶液中のβ-1,3-グルカンの粒子径が大きいことが経口摂取で抗腫瘍効果を発現しない一因であると考え，レシチンとの高圧ホモゲナイザー処理により，β-1,3-グルカンあるいはキノコ（シイタケ）エキスの微粒子化を試みた。得られた微粒子化β-1,3-グルカンのマウス腫瘍移植モデルにおける経口投与時の腫瘍増殖抑制効果を検討したところ，微粒子化により，β-1,3-グルカンおよびキノコ（シイタケ）エキスが経口投与時も腫瘍増殖抑制効果を発現することを確認した。

〔Biotherapy　17 (3) : 267-273, May, 2003〕
(Received March 28, 2003/Accepted April 10, 2003)

Antitumor Effects of Micellary Mushroom Extracts in a Murine Tumor Model

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Summary

It is known that β-1,3-glucan has antitumor effects based on activation of host immune systems, but its effects are not seen with oral administration. In this study, we prepared lecithin micelles with β-1,3-glucan or mushroom extract using high pressure homogenizer, and assessed antitumor effects of oral administration of these micelles. Micelles with both β-1,3-glucan and mushroom extract showed the inhibition of tumor growth with oral administration in murine experimental model.

Key words: Micellary mushroom extracts (MME), β-1,3-Glucan (Lentinan; LNT), Antitumor effects
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はじめに

β-1,3-グルカン（レンチナン）が宿主介在性に抗腫瘍効果を発現することは従来から多くの研究成果をもって明らかにされており[1-4]，抗腫瘍効果のみならず免疫を介した様々な病態に対しても有効であることが基礎実験で確認されている[5-8]。抗腫瘍効果が著明に観察されるモデル系の一つとしてはマウス肉腫細胞である sarcoma 180（S180）の皮下移植モデルが知られるが，このモデルにおいて，β-1,3-グルカンの静脈内投与や腹腔内投与は腫瘍を完全に退縮させる強い抗腫瘍効果を発現する一方，経口投与はまったく効果を発現しない。この理由の一つとして，β-1,3-グルカンが比較的大きな中性多糖の分子で，さらに分子同士が会合し凝集体を形成するために腸管から吸収されないことが考えられた。腸管パイエル板のM細胞は，主として抗原を取り込む機能をもつことで知られているが，様々な巨大分子，ウイルス，細菌などを取り込む。またマイクロスフェアを用いた検討で，粒子径5μ以下の粒子がM細胞を通過し腸管膜リンパ節，脾臓などの免疫組織にまで達したという報告がある[9]。β-1,3-グルカンに関しても，M細胞から吸収され免疫組織へ移行可能になれば，宿主介在性の効果を示すようになる可能性が考えられる。

そこで今回われわれは，β-1,3-グルカンの粒子径に着目し，β-1,3-グルカン溶液ならびにβ-1,3-グルカンを含有するシイタケから抽出したエキスについて高圧ホモゲナイザー処理による微粒子化を行い，マウス腫瘍移植モデルにおける経口投与時の腫瘍増殖抑制効果を検討した。

I. 対象と方法

1. 被験物質

国産生シイタケの熱水抽出により得られたエキスをシイタケ抽出エキスとして用いた。このシイタケ抽出エキスをレシチン（ツルーレシチン工業株式会社）と混和，高圧ホモゲナイザー処理することにより微粒子化シイタケ抽出エキスとした。β-1,3-グルカンはレンチナン原末（味の素株式会社）を用い調製し，同様に高圧ホモゲナイザー処理したものを微粒子化β-1,3-グルカンとした。

2. 粒度分布測定法

被験物質の平均粒子径は，株式会社堀場製作所製 LA-910 粒度分布計を用いたレーザー回折・錯乱式粒度分布測定により計測した。

3. β-1,3-グルカン濃度測定法

被験物質のβ-1,3-グルカン濃度は，水酸化ナトリウム溶液添加によるアルカリ下にコンゴーレッド溶液，リン酸を加え，β-1,3-グルカンによるコンゴーレッドの極大吸収波長のシフトを利用して，535 nm の吸光度を分光光度計にて測定することにより定量した。

4. 抗腫瘍活性評価法

in vivo にて腹腔内移植することにより継代したマウス肉腫細胞 S180 を 5～6 週齢の雌性 ICR マウス右腰背部皮下に 3×10^6/0.1 ml/head で移植した。移植翌日より被験物質を 1 日 1 回強制経口投与した。抗腫瘍活性は，腫瘍体積 [長径×短径 ^{2}÷2]，腫瘍増殖抑制率 [(1－投与群の腫瘍体積/担癌無処置群の腫瘍体積)×100] により評価した。

5. 腫瘍抗原に対する遅延型過敏反応（DTH 反応）評価法

S180 細胞を凍結融解して得た S180 の死細胞を腫瘍抗原として用いた。腫瘍移植 7 日後より被験物質の投与を開始し，9 回目の投与時すなわち腫瘍移植 15 日後，マウス右足に生理食塩水，左足に腫瘍抗原を接種し，24 時間後に足の厚みを測定，腫瘍抗原接種による足の腫れ [(24 時間後の左足厚み－接種前の左足厚み)－(24 時間後の右足厚み－接種前の右足厚み)] により評価した。

6. 統計解析

2 群間では t 検定，多群間では Dunnet one-tailed を用い，担癌無処置群に対する有意差検定を行った。

II. 結　果

1. β-1,3-グルカンの微粒子化

β-1,3-グルカン濃度 0.25 mg/ml の溶液について粒度分布を測定した。β-1,3-グルカン溶液の平均粒子径が約 200 μm，ほとんどが 20 μm 以上の粒子であった（図 1）。一方，微粒子化β-1,3-グルカン溶液の平均粒子径は約 0.1 μm，ほとんどの粒子が 1 μm 以下であった（図 2）。また，4℃あるいは室温で，微粒子化β-1,3-グルカン溶液

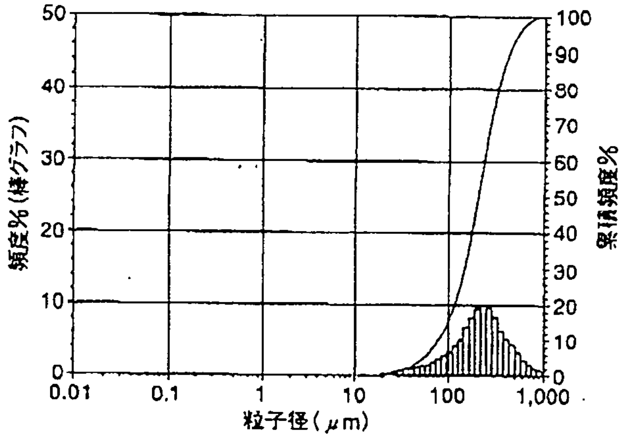

図1　β-1,3-グルカン溶液の粒度分布

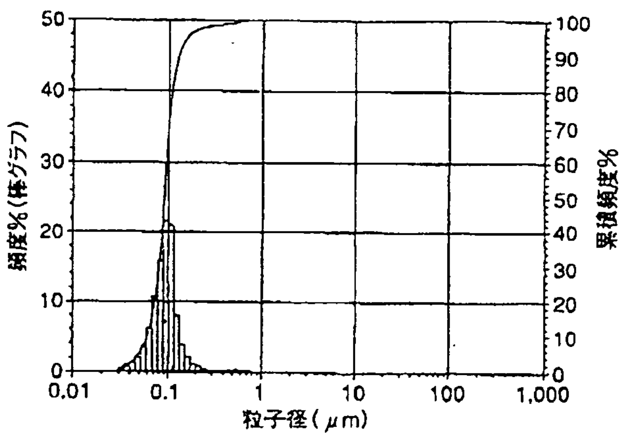

図2　微粒子化β-1,3-グルカン溶液の粒度分布

の粒度分布に3か月間変化がないことも確認した。

2.　微粒子化β-1,3-グルカンの抗腫瘍活性

S180皮下移植マウスにβ-1,3-グルカン溶液あるいは微粒子化β-1,3-グルカン溶液をβ-1,3-グルカン量として0.03 mg/headで1日1回10日間（5投2休）強制経口投与した際の腫瘍増殖抑制率を図3に示す。

β-1,3-グルカン溶液の経口投与では有意な腫瘍増殖抑制効果は認められなかったが（図3a），微粒子化β-1,3-グルカン溶液の経口投与では有意な腫瘍増殖抑制効果が認められ，その効果は腫瘍移植後16日目に最大であった（図3b）。なお微粒子化β-1,3-グルカン溶液と同濃度のレシチンに腫瘍増殖抑制効果がないことも確認した。

3.　シイタケエキスの微粒子化

β-1,3-グルカン濃度として0.15 mg/mlに調製した溶液の粒度分布を測定した。シイタケエキスはほとんどが15 μm以上の粒子で平均粒子径は約288 μmであった（図4）。一方，微粒子化シイタケエキスはほとんどが0.2 μm以下の粒子で，平均粒子径は約0.08 μmであり（図5），4℃あるいは室温で3か月間変化がないことも確認した。

4.　微粒子化シイタケエキスの抗腫瘍活性

S180皮下移植マウスにシイタケエキスあるいは微粒子化シイタケエキスをβ-1,3-グルカン量として0.03 mg/headで1日1回10日間（5投2

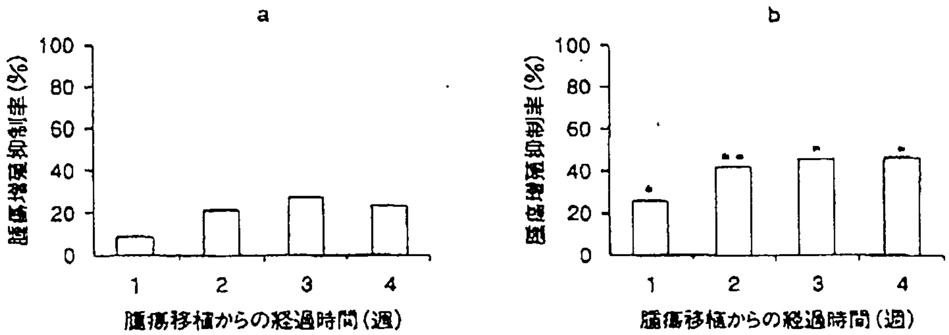

図 3 β-1,3-グルカン溶液の腫瘍増殖抑制効果
a: β-1,3-グルカン溶液
b: 微粒子化β-1,3-グルカン溶液
*: p<0.05, **: p<0.01 (担癌無処置群に対する t 検定)

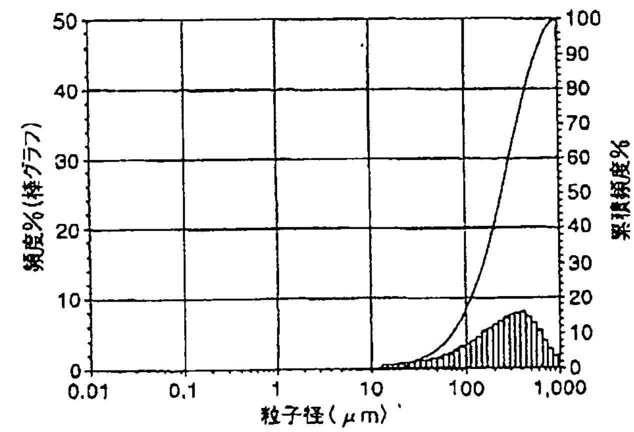

図 4 シイタケエキスの粒度分布

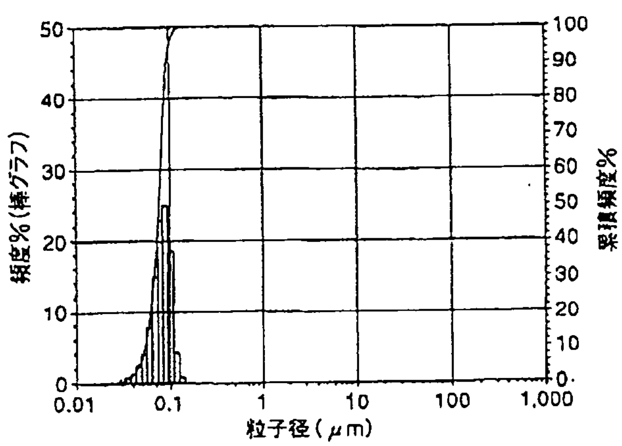

図 5 微粒子化シイタケエキスの粒度分布

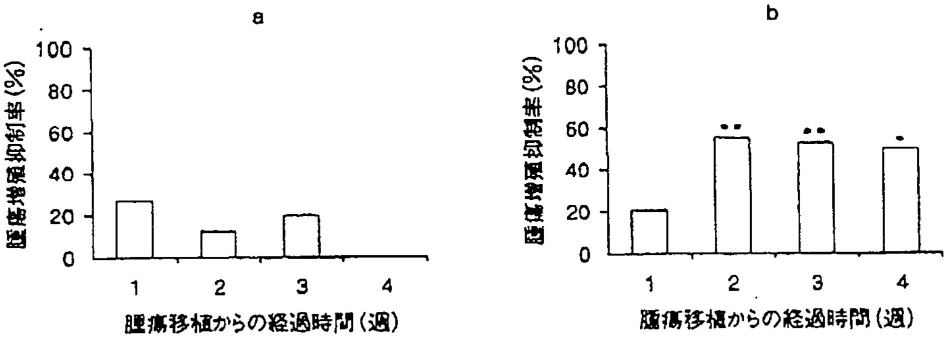

図 6 シイタケエキスの腫瘍増殖抑制効果
a：シイタケエキス
b：微粒子化シイタケエキス
*：p<0.05, **：p<0.01（担癌無処置群に対する t 検定）

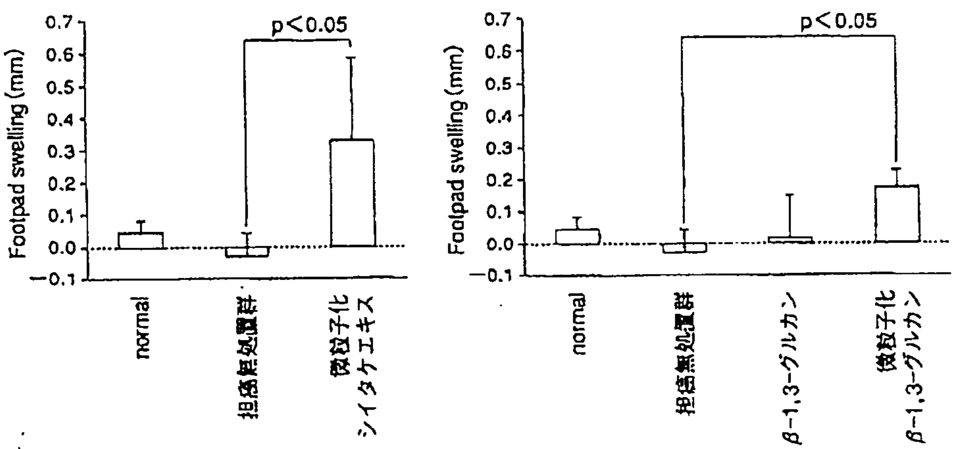

図 7 腫瘍抗原に対する遅延型過敏反応

休）強制経口投与した際の腫瘍増殖抑制率を図6に示す。

シイタケエキスの経口投与では有意な腫瘍増殖抑制効果は認められなかったが（図6a），微粒子化シイタケエキスの経口投与では有意な腫瘍増殖抑制効果が認められ，その効果は腫瘍移植後16日目に最大であった（図6b）。

5. 腫瘍抗原に対する遅延型過敏反応（DTH反応）

S180皮下移植マウスに微粒子化シイタケエキス，β-1,3-グルカン溶液および微粒子化β-1,3-グルカン溶液をβ-1,3-グルカン量として0.03 mg/headで1日1回9日間投与後に腫瘍抗原に対する遅延型過敏反応を検討した。その結果，normal群，担癌無処置群，β-1,3-グルカン溶液投与群ではDTH反応の増強が認められないのに対し，微粒子化シイタケエキスおよび微粒子化β-1,3-グルカン溶液投与群では担癌無処置群に対し有意なDTH反応の増強が認められた（図7）。

III. 考　察

マウスS180皮下移植モデルを用い腫瘍増殖抑制効果を検討した。β-1,3-グルカン溶液の経口投与は効果を発現しなかったが，今回β-1,3-グルカンの粒子径に着目し，レシチンとの高圧ホモゲナイザー処理によりβ-1,3-グルカンの粒子径を小さくしたところ，経口投与の際も抗腫瘍効果が発現することが明らかとなった。

今回用いたβ-1,3-グルカン溶液と微粒子化β-1,3-グルカン溶液の粒度分布を測定し比較したところ，微粒子化により平均粒子径は約1/2,000に縮小され，かつ粒度分布はほぼ完全に独立していた。これらの結果と粒子径5μm以下のマイクロスフェアがパイエル板から末梢のリンパ組織へ輸送されるという報告[9]から，β-1,3-グルカンは微粒子化によりパイエル板を通過し，抗腫瘍効果を示したものと考えられる。β-1,3-グルカンを含有するシイタケエキスもβ-1,3-グルカン溶液と同等の平均粒子径であったが，微粒子化により平均粒子径を約1/2,000に小さくした結果，抗腫瘍効果を発現した。

微粒子化β-1,3-グルカンと，同じβ-1,3-グルカン濃度の微粒子化シイタケエキスが同程度の腫瘍増殖抑制効果を示したことから，微粒子化シイタケエキスの有効成分はβ-1,3-グルカンであることが推察される。また，腫瘍抗原に対する遅延型過敏反応を増強したことから，微粒子化β-1,3-グルカンならびに微粒子化シイタケエキスが腫瘍増殖抑制効果発現の過程で T_{DTH} 細胞を活性化することも示唆された。

β-1,3-グルカン（レンチナン）が静脈内あるいは腹腔内投与により効果を発現する際には，まず血中のマクロファージ，好中球に接着することが知られており[10]，経口投与でも抗腫瘍効果が認められた理由としては，微粒子化β-1,3-グルカンが腸管パイエル板から吸収された後，恐らく腸管粘膜に存在するマクロファージ，樹状細胞といった免疫担当細胞と接触し，血中には移行せずに宿主の免疫系を調節し，効果を発現しているものと考えている。

現在，免疫調節能の異常を伴い発症するといわれる様々な疾患モデルに対し微粒子化シイタケエキスの作用を検討している。アトピー性皮膚炎の実験モデルに対して，予備的ではあるが血中IgEレベルの上昇ならびに皮膚炎の進行を抑制するという結果を得ている。β-1,3-グルカンがマクロファージのレドックス系に作用してTh1/Th2サイトカインバランスを調節するという報告[11]があるが，微粒子化シイタケエキスの経口投与もTh1/Th2サイトカインバランスを調節しアトピー性皮膚炎モデルに対し効果を示している可能性もあり，興味深い。

おわりに

β-1,3-グルカン含有溶液の経口投与による腫瘍増殖抑制効果を検討した。β-1,3-グルカン溶液ならびにβ-1,3-グルカンを含有するシイタケエキスは粒子径が大きく効果を示さなかったが，これら溶液をレシチンと高圧ホモゲナイザー処理し微粒子化した結果，効果を示すことが確認された。

謝辞　本研究に用いた微粒子化β-1,3-グルカン溶液，微粒子化シイタケエキス，シイタケエキスを作製していただいた味の素株式会社 生産技術センター 堀浦正俊氏，岩崎一生氏，粒度分布ならびにβ-1,3-グルカン濃度を測定していただいた味の素株式会社 食品研究所 松永佳子氏，佐藤雄士氏に感謝します。

文　献

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Title: Antitumor effect of micronized mushroom extract on tumor bearing murine model

(Biotherapy 17 (3) : 267-273, May, 2003)

Translation of p.268-273

Introduction

Up to now, by many study results, it has been shown that β-1,3-glucan (Lentinan, LNT) exerts host-mediated anti-tumor effects[1-4]. In addition to anti-tumor effects, by basic experiment, it has been confirmed that it is effective against various diseases via immunity[5-8]. Sarcoma 180 (S180) subcutaneous inoculation model of a murine sarcoma cell is known as one of model systems capable of observing the anti-tumor effects markedly. In this model, intravenous administration or intraperitoneal administration of β-1,3-glucan exerts a potent anti-tumor effect such as complete tumor regression, whereas oral administration does not exert the effect at all. It is thought that one of these reasons is that β-1,3-glucan is not absorbed through the intestinal tract since β-1,3-glucan is a molecule of relatively large neutral polysaccharide and it forms an aggregate by association between molecules. M cells in small intestinal peyer's patchs are known to have main function for uptake of antigens, and they also incorporate various large molecules, such as virus and bacteria. In addition, in an study using microsphere, there is a report that particles having 5 μ or less of diameter pass through M cells and arrive at immune tissues such as mesenteric lymph nodes and spleen [9]. With regard to β-1,3-glucan, it can be considered that β-1,3-glucan has likelihood of exerting a host mediated effect, in a case where β-1,3-glucan could be absorbed from M cells and transported to immune tissue.

Therefore, in this study, we focused on particle diameter of β-1,3-glucan, performed micronization of the β-1,3-glucan solution and the extract extracted from shiitake comprising β-1,3-glucan, and examined the inhibitory effect on tumor growth in murine tumor inoculation model in oral administration.

I. Subject and Methods

1. Test substances

An extract obtained by hot water extraction of shiitake made in Japan was used as an extract of shiitake. The extract of shiitake was converted into a micronized shiitake extract by mixing with lecithin (Tsuru Lecithin Kogyo Co., Ltd.) and treating

with high pressure homogenizer. β-1,3-glucan was prepared using bulky powder of lentinan (Ajinomoto Co., Inc.) and, similarly, converted into a micronized β-1,3-glucan using high pressure homogenizer.

2. Method of measuring particle size distribution

The mean particle diameter of the test substances was measured by a measurement using a laser diffraction/scattering particle size distribution measurement method using an LA-910 particle size distribution meter manufactured by Horiba Seisakusho Co., Ltd.

3. Method of measuring the concentration of β-1,3-glucan

The concentration of β-1,3-glucan in the test substances was quantitatively determined by adding sodium hydroxide aqueous solution so as to convert into alkaline solution, adding Congo red solution and phosphoric acid and measuring the absorbance at 535 nm with a spectrophotometer utilizing the shift of the local maximum absorption wavelength of Congo red by β-1,3-glucan.

4. Method for evaluation of anti-tumor activity

Murine sarcoma tumor cells, S180, maintained by intraperitoneal inoculation *in vivo* were subcutaneously inoclated into right groin of ICR mice, female, and 5-6-weeks-old, at 3 X 10^6/0.1 ml/head. The test substances were compulsorily and orally administrated at one time/day since the next day of the inoculation. Anti-tumor activity was evaluated with the tumor volume [maximum diameter X minimum diameter^2 $\div$ 2] and the degree of inhibition of tumor growth [(1- the tumor volume of the administred group / tumor volume of the untreated group in the tumor bearing mice) X 100].

5. Method for evaluation of a delayed type hypersensitive reaction (DTH reaction) on tumor antigen

S180 dead cells obtained by freeze-thawing of S180 cells were used as a tumor antigen. Administration of the test substances was initiated on 7th day after the tumor inoculation. At the 9th administration, i.e. on 15th day after the tumor inoculation, physiological saline was inoculated into murine right leg and tumor antigen was inoculated into left leg. 24 hours later, the thickness of each of the legs was measured, and [the delayed type hypersensitive reaction] was evaluated with the legs' swell due to the inoculation of tumor antigen [(the thickness of left leg at 24 hours later

- the thickness of left leg at before the inoculation) - (the thickness of right leg at 24 hours later - the thickness of right leg at before the inoculation)].

6. Statistical analysis

Significance Test was carried for the untreated group in the tumor bearing mice using t-test for the test between 2 groups and Dunnet one-tailed for the test between multi-groups.

II. Results

1. Micronization of β-1,3-glucan

Particle size distribution was measured for a solution comprising β-1,3-glucan concentration of 0.25 mg/ml. The mean particle diameter of the β-1,3-glucan solution was approximately 200 μm, and most of the particles were of 20 μm or more (Figure 1). On the other hand, the mean particle diameter of the micronized β-1,3-glucan solution was approximately 0.1 μm, and most of the particles were of 20 μm or less (Figure 2). Furthermore, it was also confirmed that there is no change in the particle size distribution of the micronized β-1,3-glucan solution for 3 months at 4 ℃ or room temperature.

2. Anti-tumor activity of the micronized β-1,3-glucan

Figure 3 shows the degree of inhibition of tumor growth in a case where the β-1,3-glucan solution or the micronized β-1,3-glucan solution was compulsorily and orally administered to S180 subcutaneous inoculation mice one time/day for 10 days (5-day administration and 2-day suspension) in a dose of 0.03 mg/head in terms of β-1,3-glucan amount. Significant inhibitory effect on tumor growth was not recognized by the oral administration of the β-1,3-glucan solution (Figure 3a), whereas the significant inhibitory effect on tumor growth was recognized by the oral administration of the micronized β-1,3-glucan solution and the effect was maximum on the 16th day after the tumor inoculation (Figure 3b). Furthermore, it was also confirmed that lecithin has no inhibitory effect on tumor growth at the same concentration as that of the micronized β-1,3-glucan solution.

3. Micronization of shiitake extract

Particle size distribution of a solution prepared at 0.15 mg/ml in terms of β-1,3-glucan amount was measured. Most of the particles of shiitake extract were 15 μm or more, and the mean particle diameter thereof was approximately 288 μm (Figure

4). On the other hand, most of the particles of micronized shiitake extract were 0.2 μm or less, and the mean particle diameter thereof was approximately 0.08 μm (Figure 5). It was also confirmed that there is no change in the particle size distribution for 3 months at 4 ℃ or room temperature.

4. Anti-tumor activity by micronized shiitake extract

Fig. 6 shows the degree of inhibition of tumor growth in a case where the shiitake extract or the micronized β-1,3-glucan solution was compulsorily and orally administered to S180 subcutaneous inoculation mice one time/day for 10 days (5-day administration and 2-day suspension) in a dose of 0.03 mg/head in terms of β-1,3-glucan amount. Significant inhibitory effect on tumor growth was not recognized by the oral administration of the shiitake extract (Figure 6a), whereas the significant inhibitory effect on tumor growth was recognized by the oral administration of the micronized shiitake extract and the effect was maximum on the 16th day after the tumor inoculation (Figure 6b).

5. A delayed type hypersensitive reaction (DTH reaction) on tumor antigen

After 9 days administration of the micronized shiitake extract, the β-1,3-glucan solution and the micronized β-1,3-glucan solution to S180 subcutaneous inoculation mice one time/day, in a dose of 0.03 mg/head in terms of β-1,3-glucan amount, a delayed type hypersensitive reaction on tumor antigen was examined. As a result, enhancement of DTH reaction was not recognized in the normal group, the untreated group in the tumor bearing mice, the group administered with β-1,3-glucan solution, whereas significant enhancement of DTH reaction was recognized in the group administered with micronized shiitake extract and that of β-1,3-glucan solution compared to the untreated group in the tumor bearing mice (Figure 7).

III. Discussion

An inhibitory effect on tumor growth was examined using murine S180 subcutaneous inoculation model. Oral administration of β-1,3-glucan solution did not exert the effect. On the other hand, particle diameter of β-1,3-glucan was focused in this study, thus it became apparent that the anti-tumor effect was also exhibited in the oral administration when particle diameter of β-1,3-glucan was reduced using high pressure homogenizer.

When particle size distributions of the β-1,3-glucan solution and the micronized β-1,3-glucan solution used in this study was measured and compared, the

mean particle diameter was reduced to approximately 1/2,000 by micronization and the particle size distributions was independent almost completely. According to these results and a report that microsphere having 5 μm or less of particle diameter is transported from Peyer's patch to peripheral lymphoid tissue [9], it may be thought that β-1,3-glucan passes through Peyer's patch and exerts anti-tumor effect due to micronization thereof. Although shiitake extract comprising β-1,3-glucan has the same mean particle diameter as that of β-1,3-glucan solution, the anti-tumor effect was exhibited due to reduction of the mean particle diameter to approximately 1/2,000 by micronization.

Because the micronized β-1,3-glucan and the micronized shiitake extract which are of the same β-1,3-glucan concentration exhibit the inhibitory effect on tumor growth at the same degree, it is expected that an active ingredient of the micronized shiitake extract is β-1,3-glucan. The delayed type hypersensitive reaction on tumor antigen was enhanced, thus it was also suggested that the micronized β-1,3-glucan and the micronized shiitake extract activate T_{DTH} cells in a process of exerting the inhibitory effect on tumor growth.

It was known that β-1,3-glucan (lentinan) initially adheres to a macrophage, [or] neutrophil in blood when it exerts the effect in the intravenous or intraperitoneal administration[10]. Hence, the reason that anti-tumor effect is recognized in the oral administration is considered as follows: after being absorbed through Peyer's patch in intestinal tract, the micronized β-1,3-glucan adheres to a immunocompetent cell, such as a macrophage and dendritic cell which are probably present in a mucosa of the intestinal tract, and regulates immune system of its host without transportation into blood to exhibits the effect.

An effect of the micronized shiitake extract on a model of disease which is said to be occurred along with disfunction of immunomodulatory is presently examined. There is a preliminary result that increase of the level of IgE in blood and progression of dermatitis in an experimental model of atopic dermatitis are suppressed. Interestingly, although there is a report that β-1,3-glucan affects to a redox system in a macrophage to regulate the balance of Th1/Th2 cytokine [11], there is likelihood that the oral administration of the micronized shiitake extract regulates the balance of Th1/Th2 cytokine to exert the effect on the model of atopic dermatitis.

Conclusion

An inhibitory effect on tumor growth by oral administration of a solution comprising β-1,3-glucan was examined. It was confirmed that β-1,3-glucan solution

and shiitake extract comprising β-1,3-glucan did not exert the effect because of their large particle diameter, whereas these solutions exert their effect when they are micronized together with lecithin using high pressure homogenizer.

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D2

原　著

抗腫瘍効果発現におけるβ-1,3-グルカンの粒子径の重要性

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要旨　β-1,3-グルカン（レンチナン）のもつ抗腫瘍効果は，経口投与では認められない。しかし，われわれはレンチナンを高圧ホモゲナイザー処理することにより得た微粒子化体が，経口投与でも抗腫瘍効果を発現することを見いだした。今回われわれは，様々な粒子径のβ-グルカンと高圧ホモゲナイザー処理による微粒子化体との比較を，マウス腫瘍移植モデルにおける腫瘍増殖抑制効果により検討した。その結果，β-グルカンはその粒子径にかかわらず，腹腔内投与では腫瘍増殖抑制効果を発現したが，経口投与時には腫瘍増殖抑制効果を発現せず，高圧ホモゲナイザー処理による微粒子化体のみがいずれの投与経路においても有効であることが確認された。

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The Importance of Size for Anti-Tumor Effects of β-Glucan

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Summary

It is known that β-1,3-glucan has anti-tumor effects based on activation of host immune systems, but these effects are not seen with oral administration. We prepared lecithin micelles with β-1,3-glucan using a high pressure homogenizer, and assessed anti-tumor effects of oral administration of these micelles. Recently, we prepared various sizes of β-glucan without using the high-pressure homogenizer, and compared them to lecithin micelles with β-glucan for anti-tumor effects using a murine experimental model. In the results, all β-glucan showed anti-tumor effects by intraperitoneal administration, but only lecithin micelles with β-glucan did by oral administration.

Key words: Lecithin micelles, β-1,3-glucan (Lentinan, LNT), Anti-tumor effects

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はじめに

β-1,3-グルカン（レンチナン，LNT）が宿主介在性に抗腫瘍効果を発現することは従来から多くの研究成果をもって明らかにされており[1-4]，抗腫瘍効果のみならず免疫を介した様々な病態に対しても有効であることが基礎実験で確認されている[5-8]。LNTの抗腫瘍効果は，マウス肉腫細胞Sarcoma180（S180）の皮下移植モデルを用いて数多く検討された。このモデルにおいて，LNTは静脈内投与や腹腔内投与では腫瘍完全退縮を含む強い抗腫瘍効果を発現する一方で，経口投与では効果をまったく発現しない。この理由の一つとして，LNTが分子同士で会合し凝集体を形成するために腸管から吸収されないことが考えられた。腸管パイエル板のM細胞は，主として抗原を取り込む機能をもつことで知られているが，様々な巨大分子，ウイルス、細菌などを取り込む。またマイクロスフェアを用いた検討で，粒子径5μm以下の粒子がM細胞を通過し腸管膜リンパ節，脾臓などの免疫組織にまで達したという報告がある[9]。LNTに関しても，M細胞から吸収され免疫組織への移行が可能になれば，宿主介在性の効果を示すようになる可能性が考えられた。このことからわれわれはLNTの粒子径に着目し，LNTの微粒子化体を作製，マウス腫瘍移植モデルにおいて経口投与時にも腫瘍増殖抑制効果を発現することを確認した[10]。さらに，LNTの精製元であるシイタケ抽出エキスについても同様の微粒子化処理を行い，経口投与での腫瘍増殖抑制効果を確認し，その主たる有効成分がLNTであることも確認した。

最近われわれは，溶液調製時の濃度によりLNT溶液の粒子径が異なることを見いだした。そこで，様々な粒子径のLNT溶液について抗腫瘍活性の有無を検討し，微粒子化シイタケ抽出エキスおよび微粒子化LNTと比較検討した。

I. 対象と方法

1. 被験物質

LNT溶液はLNT原末（味の素株式会社）を蒸留水に懸濁し120℃，20分高圧処理により調製した。LNT溶液をレシチン溶液と混合し，高圧ホモゲナイザー処理したものを微粒子化LNT（M-LNT）溶液とした。また，シイタケ抽出エキスの微粒子化体としては「ミセラピスト® 超微粒子β-グルカン」（MME：味の素株式会社）を用いた。

LNTの金コロイド標識には，MONOAMINO NANOGOLD LABELING REAGENT（Nanoprobes社）を用いた。

2. 粒度分布測定法

被験物質の平均粒子径は，株式会社堀場製作所製LA-910粒度分布計を用いたレーザー回折・散乱式粒度分布測定により計測した。

3. LNT濃度測定法

被験物質のLNT濃度は，水酸化ナトリウム溶液添加によるアルカリ状態でコンゴーレッド溶液と混合，リン酸を加えた後，LNTによるコンゴーレッドの極大吸収波長のシフトを利用して，535 nmの吸光度を分光光度計にて測定することにより定量した。

4. 抗腫瘍活性評価法

*in vivo*における腹腔内移植により継代したマウス肉腫細胞S180を，5～6週齢の雌性ICRマウス右腹背部皮下に3×10^6/0.1 ml/headで移植した。移植翌日より被験物質を1日1回強制経口投与あるいは腹腔内投与した。抗腫瘍活性は，腫瘍体積［長径×短径^2÷2］，腫瘍増殖抑制率［(1－投与群の腫瘍体積/担癌無処置群の腫瘍体積)×100］により評価した。

5. 小腸パイエル板からの取り込み観察

雄性ddyマウスを麻酔下にて開腹し，パイエル板を含むように小腸に3 cmのループを作製した。LNT溶液の金コロイド標識体あるいは金コロイド標識M-LNTをLNT量として0.12 mg注入し，2時間後にサンプリング、凍結切片を作製，銀増感により可視化した。電子顕微鏡による観察の際は，金コロイド標識M-LNTを0.03 mg注入した。

6. 統計解析

担癌無処置群と各処置群の2群間でt検定を行い，有意差検定を行った。

II. 結　果

1. LNT溶液の調製

LNT濃度2 mg/mlで調整した溶液の平均粒子径は約130μmであった。この溶液を遠心分離

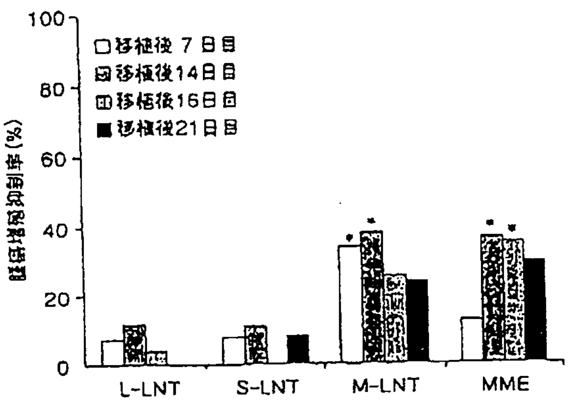

図1　0.03 mg/head/day 経口投与による抗腫瘍活性
*: $p<0.05$（担癌無処置群に対する t 検定）

(8,000 G, 10 min) した結果，上清に約60%のLNTが存在した。沈殿を再度蒸留水に懸濁したところ溶液の平均粒子径は約130 μm であり，これを大粒子 LNT (L-LNT) とした。LNT 濃度 0.2 mg/ml で調製した場合にも，平均粒子径は約130 μm であったが，遠心分離 (8,000 G, 10 min) により得られた上清は原液の85%に当たるLNTを含み，粒子径は測定可能範囲以下であった。この溶液を水溶性 LNT (S-LNT) とした。一方，M-LNT および MME の平均粒子径は約0.4 μm，ほとんどの粒子が1 μm 以下であった。

また，いずれの LNT 溶液についても，4℃あるいは室温で，LNT 溶液の粒度分布に3か月間変化がないことも確認した。

2. 各 LNT 溶液の経口投与による抗腫瘍活性

S180 皮下移植マウスに L-LNT，S-LNT，M-LNT あるいは MME を LNT 量として 0.03 mg/head で，腫瘍移植翌日より1日1回10日間（5投2休）強制経口投与した。その際の腫瘍増殖抑制率を図1に示す。L-LNT，S-LNT には抗腫瘍活性は認められなかったが，M-LNT ならびに MME はいずれも有意な抗腫瘍効果を示した。

各溶液について 0.03 mg/head が至適投与量ではない可能性も考えられたため，L-LNT, S-LNT, MME を LNT 量として 0.01，0.09 mg/head で1日1回10日間（5投2休），強制経口投与した。その際の腫瘍増殖抑制率を図2に示す。

L-LNT あるいは S-LNT の経口投与では有意な腫瘍増殖抑制効果は認められなかったが（図2a, b），

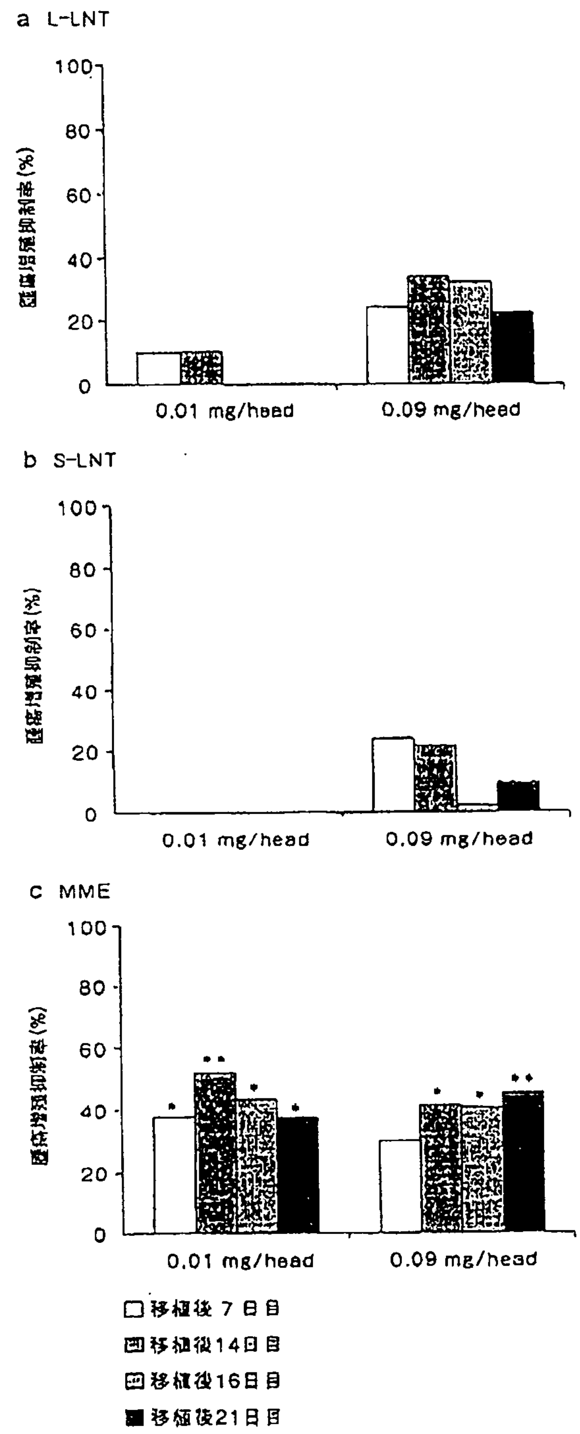

図2　0.01 および 0.09 mg/head/day 経口投与による抗腫瘍活性
*: $p<0.05$, **: $p<0.01$
（担癌無処置群に対する t 検定）

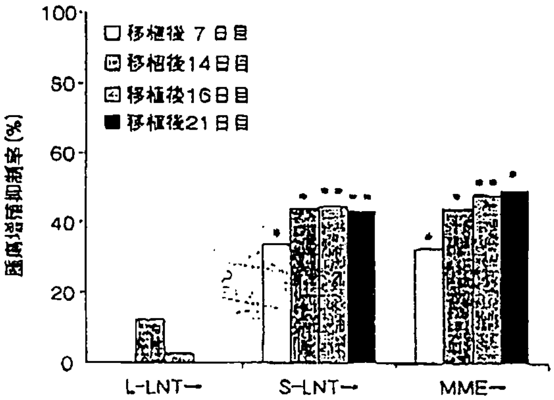

図 3　MME の抗腫瘍活性に与える LNT の影響
*: $p<0.05$, **: $p<0.01$
(担癌無処置群に対する t 検定)

MME の経口投与ではいずれの投与量においても有意な腫瘍増殖抑制効果が認められた (図 2c)。

3. MME の抗腫瘍活性に与える LNT の影響

微粒子化処理していない LNT は経口投与で無効であった。その原因の一つとして考えられる取り込みについて調べるため，MME を 0.03 mg/head で経口投与する 10 分前に L-LNT，S-LNT あるいは MME を 0.03 mg/head で経口投与し，MME の腫瘍増殖抑制効果に与える影響を検討した。図 3 に示すように，MME は 2 回投与しても有意な腫瘍増殖抑制効果を示したのに対し，先に L-LNT を投与した際は MME の腫瘍増殖抑制効果は完全に抑制された。一方，S-LNT 前投与は MME の腫瘍増殖抑制効果にまったく影響を与えなかった。

4. 各 LNT 溶液の腹腔内投与による抗腫瘍活性

各 LNT 溶液が，本来の作用を有しているか否か検討するため，各溶液を LNT 量として 0.01，0.03，0.09 mg/head で 1 日 1 回 10 日間 (5 投 2 休)，腹腔内投与した。その結果，いずれの LNT 溶液も高い腫瘍増殖抑制率を示し (図 4)，腹腔内投与時の作用に差異はないことが確認された。

5. 小腸パイエル板からの取り込み

非微粒子化 LNT 溶液については，パイエル板上皮への接着および内部への取り込みがほとんど認められなかった (図 5a) のに対し，M-LNT では，パイエル板上皮への接着および内部への取り込みが認められた (図 5b)。電子顕微鏡により，

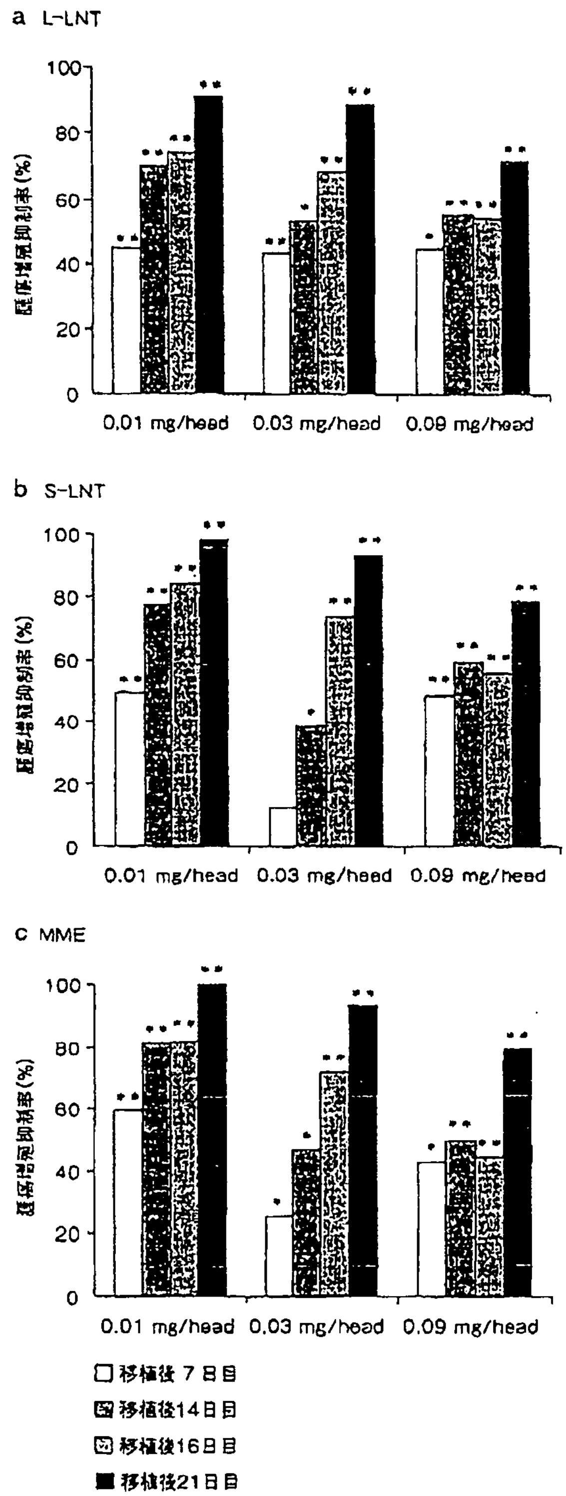

図 4　各 LNT 溶液の腹腔内投与による抗腫瘍活性
*: $p<0.05$, **: $p<0.01$
(担癌無処置群に対する t 検定)

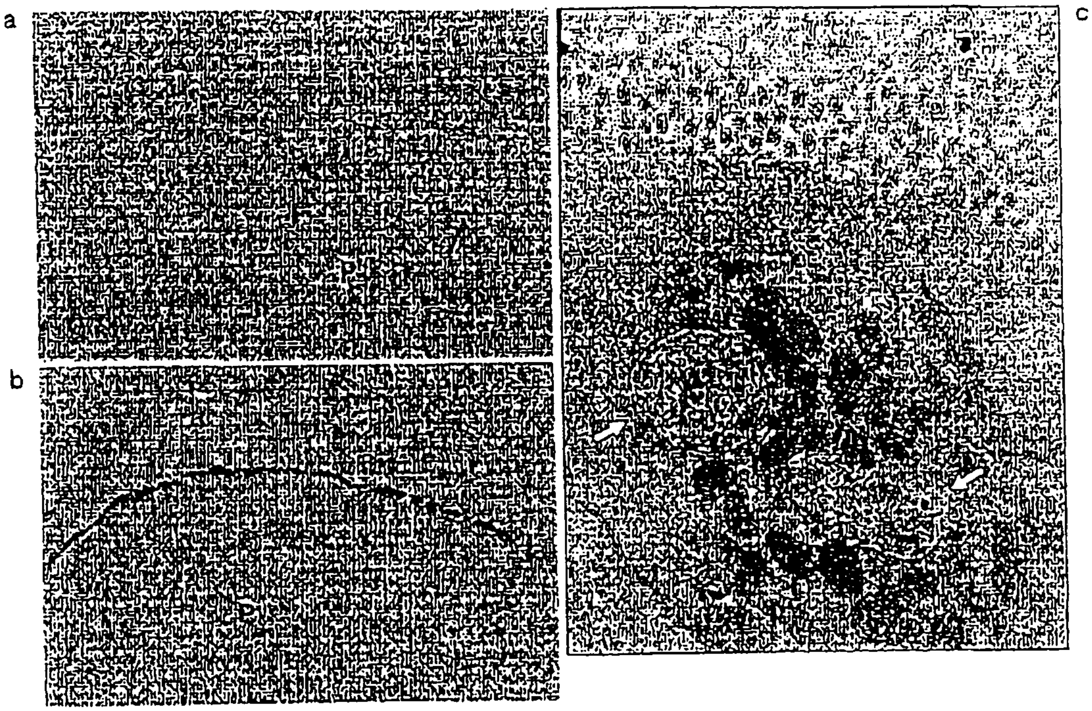

図5 小腸パイエル板からの取り込み
P：パイエル板

M-LNT がパイエル板上皮細胞の空胞に存在することを観察した（図5c）。

III. 考 察

粒子径の異なるLNT溶液を調製し，微粒子化処理を行ったLNT溶液との比較をマウス腫瘍移植モデルにおける腫瘍増殖抑制効果により検討した。

その結果，いずれの粒子径のLNT溶液も腹腔内投与では高い抗腫瘍効果を発現し，LNTとしての作用は保持しているものと考えられた。LNTの分子量と腹腔内投与時の抗腫瘍活性に関しては，S-LNTのうち分子量6,000未満のものはまったく効果を発現せず，分子量6,000，16,200のものは効果を発現したとの報告がある[11]。また，水に不溶な画分については分子量30,000以上で，いずれも高い効果を発現することが示されている。これらのことから，今回用いたLNTのうちS-LNTは分子量6,000以上の水溶性画分で，L-LNTは分子量30,000以上のものであったことが予想される。微粒子化処理を行ったLNTについては，遠心分離の操作は行っておらず，様々な分子量のLNTを含有していた可能性が高い。

一方，経口投与ではL-LNTもS-LNTも抗腫瘍活性はなく，微粒子化処理したLNTのみが抗腫瘍効果を発現した。粒子径5 μm以下のマイクロスフェアがパイエル板から末梢のリンパ組織へ輸送されるという報告[9]があり，経口投与されたLNTの取り込みにおいても，小腸パイエル板のM細胞が中心的役割を果たしているのではないかと考えられる。今回，小腸ループへの標識体注入を行ったところ，微粒子化処理したLNTについてパイエル板への取り込みが確認された。S-LNTは粒子径が測定限界以下でM-LNTよりも小さな粒子であるため，小腸パイエル板からの取り込みは可能であると考えられる。また，腹腔内投与ではS-LNTも抗腫瘍効果を示したことから，S-LNTが経口投与においても抗腫瘍効果を発現する可能性が考えられたが，実際には無効であった。この原因について考察するため，L-LNT，S-

LNT あるいは MME を経口投与し，10 分後に MME を経口投与，MME の腫瘍増殖抑制効果に及ぼす影響を検討した。その結果，L-LNT が MME の腫瘍増殖抑制効果を抑制したのに対し，S-LNT は無影響であった。L-LNT については，腸管からの取り込みの第一段階では MME と同じ経路を介することが考えられ，その結果，MME の小腸パイエル板からの取り込みが阻害されたものと考えられる。一方，S-LNT について，腹腔内投与では効果を発現し経口投与では粒子径が小さいにもかかわらず無効であった理由として，腸管と全身では LNT に対する免疫反応に違いがある可能性が考えられる。今後，小腸パイエル板を中心に，M-LNT の取り込みそのものに対する L-LNT あるいは S-LNT の影響について組織科学的検討を考えている。

おわりに

LNT 含有溶液の経口投与ならびに腹腔内投与による腫瘍増殖抑制効果を検討した。腹腔内投与では粒子径にかかわらず強い効果を示したが，経口投与では微粒子化処理を行ったもののみが効果を示した。微粒子化処理していない LNT 溶液は，粒子径の大小にかかわらず効果を示さなかった。

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0914-2223/05/¥500/論文/JCLS

Title: The importance of Size for Anti-Tumor Effects of β-glucan

(Biotherapy 19 (3) : 273-278, May, 2005)

Translation of p.274-278

Introduction

Up to now, by many study results, it has been shown that β-1,3-glucan (Lentinan, LNT) exerts host-mediated anti-tumor effects[1-4]. In addition to anti-tumor effects, by basic experiment, it has been confirmed that it is effective against various diseases via immunity[5-8]. The anti-tumor effects of LNT were greatly studied using a subcutaneous inoculation model of a murine sarcoma cell, Sarcoma 180 (S180). In this model, LNT exerts strong anti-tumor effects such as complete tumor regression in case of intravenous administration or intraperitoneal administration, whereas LNT does not exert an effect at all in case of oral administration. It is thought that one of these reasons is that LNT is not absorbed through the intestinal tract since it forms an aggregate by association between molecules.

M-cells in intestinal peyer's patchs are known to have the main function of absorption of antigens, they absorb various large molecules, virus and bacteria. There is also a report that in the study using microsphere, particles of 5 micro meters or less in size passed the M-cells to reach immune tissues such as mesenteric lymph nodes and spleen [9]. With regard to the LNT, it was thought that there is a possibility of showing the host-mediated effect if the LNT can be absorbed into the M cells and then be transferred to immune tissues. For this reason, focusing on the particle size of the LNT, a micronized LNT was constructed, and then it was confirmed that the micronized LNT exerted inhibitory effects on tumor growth at oral administration in murine tumor inoculation model [10]. In addition, with regard to the extract of shiitake which was the source of purified LNT, it was subjected to the same micronized treatment and the anti-tumor effects thereof were confirmed in case of the oral administration, and then it was confirmed that the main active ingredient thereof was the LNT.

Recently, it was found that the particle size of the LNT solution was different from the density at the preparation of the solution. Thus, with regard to the LNT solutions having the various particle sizes, it was confirmed whether or not they had anti-tumor effects and then they were compared to the micronized shiitake extract and LNT.

I. Subject and Methods

1. Test substances

A bulky powder of LNT (Ajinomoto Co., Inc.) was suspended in distilled water and then the resulting suspension was subjected to high-pressure treatment at 120℃ for 20 minutes to prepare LNT solution. After mixing the LNT solution with a Lecitin solution, the mixture was subjected to high-pressure homogenized treatment to be a micronized LNT solution (M-LNT). Further, "Micellapist Superfine Dispersed BETA-Glucan" (MME : Ajinomoto Co., Inc.) was used as a micronized shiitake extract.

MONOAMINO NANOGOLD LABELING REAGENT (Nanoprobes, Inc.) was used as a gold labeling reagent for labeling the LNT.

2. Particle size distribution measuring method

The mean particle size of the test substances were measured by a laser diffraction/scattering particle size distribution measurement method using an LA-910 particle size distribution meter manufactured by Horiba Seisakusho Co., Ltd.

3. LNT concentration measuring method

LNT concentrations of test substances were measured by followings:

The test substances were mixed with a Congo red solution under alkaline condition that was formed by adding sodium hydroxide aqueous solution. After adding phosphoric acid, utilizing the shift of the local maximum absorption wavelength of Congo red by the LNT, the LNT concentration was quantitatively determined by measuring the absorbance at 535 nm with a spectrophotometer.

4. Anti-tumor activity measuring method

Sarcoma 180 tumor cells maintained by intraperitoneal inoculation in vivo were subcutaneously inoculated into a right groin of ICR mice (female, from 5 to 6 week-old) at 3×10^6/0.1ml/head. The test substances were orally or intraperitoneally administered once daily (to the mice) since the next day of the inoculation. Anti-tumor activity were evaluated by tumor volume [(tumor maximum diameter) × (tumor minimum diameter)^2 ÷ 2], and degree of inhibition of tumor growth [(1 − (tumor volume of administrated group) ÷ (tumor volume of the untreated group)) × 100].

5. Observation of absorption from the Peyer's patchs of the small intestine

A male ddy mice were given celiotomy under anesthesia and then 3 cm length of

loop so as to include Peyer's patchs was constructed in a small intestine. The LNT solution or M-LNT, labeled with gold-colloid [the amount of the LNT was 0.12 mg] was injected [into the loop] and then 2 hours after injection, a sample [of the loop] was collected. A frozen section thereof was prepared and then the resulting frozen section was visualized by silver-enhancement. At the time of observation by electron microscopy, 0.03 mg of the M-LNT labeled with gold-colloid was injected.

6. Statistical analysis

T-test was performed between group in the tumor bearing mice and each treatment group, and then significant test was performed.

II. Results

1. Preparation of LNT solution

The mean particle size of the solution adjusted to LNT concentration of 2 mg/ml was approximately 130 μm. The solution was subjected to centrifugation (8,000G, 10min), so that there was approximately 60% of LNT in the supernatant. When the precipitation was suspended in distilled water again, the mean particle size of the solution was approximately 130 μm. This was referred to as the large particle LNT (L-LNT). In case of the preparation at LNT concentration of 0.2 mg/ml, the mean particle size was also approximately 130 μm, and, however, the supernatant obtained through centrifugation (8,000G, 10min) contained LNT corresponding to 85% of stock solution, and the mean particle size was below the possible range of measurement. This solution was referred to as the water-soluble LNT (S-LNT). On the other hand, the mean particle sizes of M-LNT and MME was approximately 0.4 μm, and most of the particles were below 1 μm.

In addition, with regard to any LNT solutions, it was confirmed that the size distributions of LNT solutions do not change for 3 months at 4 °C or room temperature.

2. Anti-tumor activity of various LNT solutions by oral administration

L-LNT, S-LNT, M-LNT or MME was orally administered to S180 subcutaneous inoculation mice one time/day for 10 days (5-day administration and 2-day suspension) in a dose of 0.03 mg/head in terms of LNT amount from next day after transplant of the tumor. Figure 1 shows the then degree of inhibition of tumor growth. L-LNT and S-LNT did not show an anti-tumor activity. However, both of M-LNT and MME showed significant anti-tumor effects.

With regard to each LNT solutions, in view of a possibility that 0.03 mg/head

was not an optimum dose, L-LNT, S-LNT, MME was orally administered one time/day for 10 days (5-day administration and 2-day suspension) in a dose of 0.01, 0.09 mg/head in terms of LNT amount. Figure 2 shows the then degree of inhibition of tumor growth.

In case of the oral administration of L-LNT or S-LNT, significant inhibitory effect on tumor growth was not shown (Figure 2a, b). However, in case of the oral administration of MME at any dose, significant inhibitory effect on tumor growth was shown (Figure 2c).

3. Influence of LNT on anti-tumor activity of MME

In the oral administration, LNT without fine pulverization treatment was not effective. For evaluation for incorporation, which might be one of the causes, L-LNT, S-LNT or MME was orally administered in a dose of 0.03 mg/head 10 min prior to the oral administration of MME at 0.03 mg/head, so that influence on anti-tumor activity of MME was studied. As shown in Figure 3, when MME was administered twice, significant inhibitory effect on tumor growth was shown. On the contrary, when L-LNT was administered in advance, the inhibitory effect of MME on tumor growth was completely suppressed. On the other hand, pre-administration of S-LNT did not affect the inhibitory effect of MME on tumor growth.

4. Anti-tumor activity of various LNT solutions by intraperitoneal administration

For studying whether each LNT solutions have inherent action or not, each solutions was intraperitoneally administered one time/day for 10 days (5-day administration and 2-day suspension) in a dose of 0.01, 0.03, 0.09 mg/head in terms of LNT amount. As a result, all LNT solutions showed a high degree of inhibition of tumor growth (Figure 4), and it was confirmed that there was no difference in action in intraperitoneal administration.

5. Incorporation into small intestinal Peyer's patch

In case of LNT solution without fine pulverization treatment, LNT was little adhered on epithelium of Peyer's patch and LNT was little incorporated into the internal region of Peyer's patch (Figure 5a). On the contrary, in case of M-LNT, it was adhered on epithelium of Peyer's patch and it was incorporated into the internal region of Peyer's patch (Figure 5b). Using an electron microscope, presence of M-LNT was observed in vacuole of epithelial cell of Peyer's patch.

III. Discussion

The LNT solutions having the different particle sizes were prepared respectively and anti- tumor effects thereof were compared to that of the micronized LNT solution in the murine experimental model of tumor implantation.

In the results, the LNT solutions having any of the particle sizes showed high anti -tumor effects in case of the intraperitoneal administration. Therefore it was thought that they retained function as the LNT. With regard to (the correlation between) the molecular weight of the LNT and the anti-tumor effects at intraperitoneal administration, there is a report that the S-LNT having the molecular weight of less than 6000 showed no effect whereas it having the molecular weight of 6000 and 16200 showed effects[11)]. With regard to the water-insoluble fraction, it is also indicated that any of the water-insoluble fractions having the molecular weight of 30000 or more showed high effects. From these reports, it is anticipated that among the LNT which were used in the present study, the S-LNT in the water-soluble fraction had the molecular weight of 6000 or more, and the L-LNT had the molecular weight of 30000 or more. With regard to the micronized LNT, it is highly possible that the micronized LNT included the LNT with various molecular weights because it was not subjected to centrifugation.

On the contrary, in case of the oral administration, both the L-LNT and S-LNT had no anti-tumor effects but only the micronized LNT showed anti-tumor effects. There is a report that microsphere of particle size of 5 micro meter or less is transported from peyer's patchs to peripheral lymphatic tissue[9)]. Thus, it is thought that M cells in peyer's patchs of the small intestine play a central role in the absorption (incorporation) of the orally-administrated LNT. In the present study, with regard to the micronized LNT, it was confirmed that the micronized LNT was absorbed by peyer's patchs when the labeled LNT(s) were injected into the intestine loop. Since the particle size of the S-LNT, which is below the detection limit, is smaller than that of M-LNT, it is thought that the absorption of the S-LNT by peyer's patchs of the small intestine is possible. The S-LNT also showed anti-tumor effects in case of the intraperitoneal administration and thus it was thought that there was a possibility of showing the anti-tumor effect in case of the oral administration of S-LNT, however, it actually was not effective. To discuss the cause (of the above mentioned result), the L-LNT, S-LNT or MME were orally-administered respectively, then 10 minutes after administration, the MME was administered to each of them to study the influence on inhibitory effects on tumor growth by the second MME administration . In the result, the L-LNT suppressed inhibitory effects on tumor growth of the MME whereas the S-LNT did not influence.

With regard to the L-LNT, it is thought that the L-LNT follows the same pathway as MME in the first step of the absorption from the intestinal tract then in the result, it is thought that the absorption of the MME by the peyer's patchs of the small intestine was inhibited. On the contrary, with regard to the S-LNT, it is thought that there is the difference of the immune reaction against the LNT between the intestinal tract and whole body as a possible reason for the S-LNT with small particle size showed effects in case of the intraperitoneal administration whereas it showed no effect in case of the oral administration.

Hence, it is thought that histological study will be performed about the influence of the L-LNT or S-LNT for the absorption of the M-LNT.

Conclusion

The inhibitory effects on tumor growth by the intraperitoneal or oral administration of the solution including the LNT were studied. In case of the intraperitoneal administration, the solution having any particle size showed strong effects whereas in case of the oral administration, only the micronized solution showed the effects. Whether the particle size was large or small, the LNT solution without micronized treatment showed no effect.

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Biomedical Research 28 (2) 71-77, 2007

Potentiation of intestinal immunity by micellary mushroom extracts

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ABSTRACT

Mushroom (shiitake) extracts were dispersed with lecithin micelles to prepare superfine particles (0.05 to 0.2 μm in diameter) of β-1,3-glucan (micellary mushroom extracts). When mice were fed with these micelles of β-glucan (0.75 mg/day/mouse, smaller amounts of β-glucan), the number of lymphocytes yielded by the small intestine increased by up to 40%. More interestingly, the ratio of $CD8\alpha\beta^+TCR\alpha\beta^+$ cells/$CD8\alpha\alpha^+TCR\alpha\beta^+$ cells increased prominently. In parallel with this deviation in the distribution of lymphocyte subsets, tumor cytotoxicity against P815 cells and cytokine productions were also augmented. In other words, phylogenetically developed lymphocytes ($CD8\alpha\beta^+$, $TCR\alpha\beta^+$) were much more effectively activated by the oral administration of micellary β-glucan. These results suggest that smaller amounts of micellary β-glucan might be useful for the potentiation of intestinal immunity.

It is empirically known that mushrooms, especially components of β-glucan, are good for our health (2, 6, 10, 14, 17, 21) and sometimes show anti-tumor effects in animal models and humans (3, 4, 15). However, such anti-tumor effects of crude mushroom extracts were limited in our preliminary experiments. To overcome this, we prepared superfine particles of β-1,3-glucan (*i.e.*, mushroom extracts) dispersed with lecithin micelles using a high-pressure emulsifier in this study. These mushroom (shiitake) extracts were then used to examine whether oral administration has the potential to induce the augmentation of intestinal immunity in mice. Cumulative evidence has revealed that the augmentation of intestinal immunity is extremely important for immunological tolerance, anti-tumor effects, innate immunity against intracellular pathogens, etc. (1, 7, 8, 13, 18). The present results indicate that micellary mushroom extracts might have such potential via the augmentation of intestinal immunity, especially in the small intestine.

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MATERIALS AND METHODS

Mice. C57BL/6 (B6) mice at the age of 8–10 weeks were used in this study. The mice were maintained in the animal facility of Niigata University (Niigata, Japan). All mice were fed under specific pathogen-free conditions. All experimental procedures were approved by the Committee on Animal Research of Niigata University.

Oral administration of shiitake extracts. Shiitake mushrooms were broken down with a colloid mill and boiled at 95°C for 3 h in hot water, and the resulting extracts were filtered. These extracts were then mixed with lecithin and dispersed at 1500 kgf/cm^2 with a high-pressure emulsifier (Ajinomoto Co. Inc., Kawasaki, Japan). Particles of β-1,3-glucan in the shiitake extracts (10 ~ 1000 μm) became smaller up to particle size of 0.05 to 0.2 μm (Fig. 1). The major component of the superfine particles was

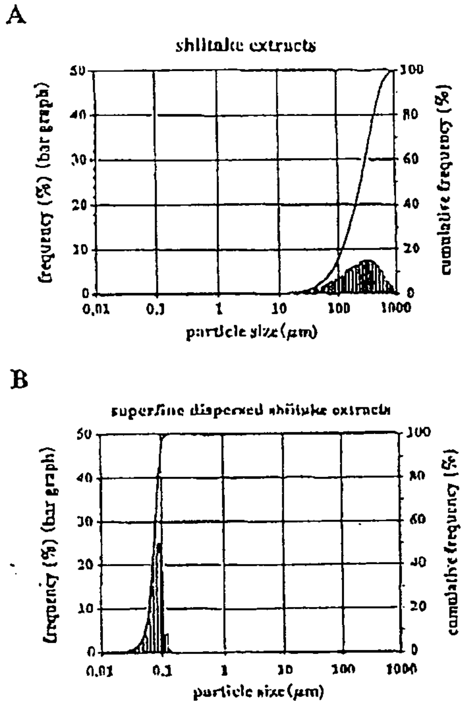

Fig. 1 Particle size distribution of the shiitake extract. A. Crude shiitake extracts. B. Micellary shiitake extracts. Shiitake extracts were mixed with lecithin and were dispersed at 1500 kgf/cm² using a high-pressure emulsifier. Diameter of particles was determined by a laser diffraction/scattering particle size distribution meter.

β-glucan. The concentration of β-glucan was quantitatively determined using Congo red solution by measuring the absorbance at 535 nm with a spectrophotometer. These micellary extracts were diluted with drinking water, which mice drank from a bottle for 2 weeks (0.75 mg/day/mouse).

Cell preparation. Mice anesthetized with ether were sacrificed by total bleeding from the incised axillary artery and vein. The organs to be used for the experiments were removed and lymphocytes were obtained as follows. Hepatic lymphocytes were isolated by a previously described method (22). Briefly, the liver was pressed through 200-gauge stainless steel mesh and suspended in Eagle's MEM medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 5 mM Hepes and 2% heat-inactivated newborn calf serum. After being washed once with the medium, the cells were fractionated by centrifugation in 15 mL of 35% Percoll solution (Amersham Biosciences, Uppsala, Sweden) for 15 min at 440 × *g*. The resulting pellet was resuspended in erythrocyte lysing solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 1 mM EDTA-Na, and 17 mM Tris, pH 7.3). Splenocytes were obtained by forcing the spleen through 200-gauge stainless steel mesh. Splenocytes were treated with 0.2% NaCl solution to remove RBC.

Intraepithelial lymphocytes (IEL) were collected from the small intestine according to a previously described method (19). Briefly, the small intestine was removed and flushed with PBS to eliminate luminal contents. The mesentery and Peyer's patches were then resected. The intestine was opened longitudinally and cut into 1-2 cm fragments. These fragments were incubated for 15 min in 20 mL Ca^{2+}-and-Mg^{2+}-free Dulbecco's PBS containing 5 mM EDTA, in a 37°C shaking-water bath. The supernatant was then collected. The cell suspensions were collected and centrifuged in a discontinuous 40%/80% Percoll gradient at 830 × *g* for 25 min. Cells from the 40%/80% interface were collected.

Immunofluorescence tests. Standard flow cytometric analysis was performed as previously described (16). FITC-conjugated anti-CD3 (145-2C11), anti-CD8α (53-6.7), PE-conjugated anti-NK1.1 (PK136), anti-IL-2Rβ (TM-β1), anti-CD45R/B220 (RA3-6B2), anti-CD4 (PM4-5), anti-CD8β (53-5.8), biotin-conjugated anti-TCRαβ (H57-597), anti-TCRγδ mAbs (GL3) and their isotype controls were purchased from BD PharMingen (San Diego, CA). Biotin-conjugated reagents were developed with Tricolor conjugated streptavidin (Caltag Lab, San Francisco, CA).

Cytotoxicity assay. Cytotoxicity assay was performed as previously described (5). YAC-1 and P815 target cells were labeled with sodium [^{51}Cr] chromate (NEN Life Science Products, Boston, MA) for 2 h and washed three times with RPMI-1640 medium supplemented with 10% fetal calf serum (FCS). P815 target cells were preincubated with anti-TCRαβ (H57-597, 1 μg/mL), anti-TCRγδ (GL3, 1 μg/mL), and anti-CD3ε (145-2C11, 1 μg/mL), respectively. Effector cells were serially diluted and mixed with [^{51}Cr]-labeled target cells (1×10^4 cells) in a 96-well U-bottomed microculture plate. The plate was centrifuged and incubated for 4 h at 37°C. At the end of the culture, 100 mL of supernatant was counted in a gamma counter.

Quantification of cytokines. Sera obtained from each mouse were used to detect the concentration of in-

terleukin-2 (IL-2) and tumor necrosis factor α (TNFα) using Cytometric Bead Array (CBA) kits (BD Biosciences, San Jose, CA) according to the manufacturer's instructions. The CBA technique is based on microparticles matched with antibodies, in which the particles are dyed to different fluorescence intensities. Following acquisition of sample data using a FACScan (BD Biosciences), the sample results were generated in graphical and tabular formats using BD CBA Analysis Software (BD Biosciences).

Statistical analysis. The difference between the values was determined by Student's *t*-test.

RESULTS

Immunopotentiation in the small intestine by the administration of β-glucan

Mice drank pure water or water with shiitake extracts (*i.e.*, micellary β-glucan) for 2 weeks and the numbers of lymphocytes yielded by the liver, spleen and small intestine were enumerated (Fig. 2). The numbers of lymphocytes in the liver and spleen were unchanged by the oral administration of β-glucan, whereas that in the small intestine (intraepithelial lymphocytes, IEL) increased up to 40% by the administration of β-glucan ($n = 4$, $p < 0.05$).

To determine the distribution of lymphocyte subsets in various immune organs, two-color staining for CD3 and IL-2Rβ and that for CD3 and NK1.1 were then conducted (Fig. 3A). Mice fed with or without β-glucan were examined on day 14. In the liver, $CD3^-$IL-$2R\beta^+$ were NK cells, $CD3^{int}$IL-$2R\beta^+$ were extrathymic T cells and $CD3^{high}$IL-$2R\beta^-$ were conventional T cells of thymic origin (20). NK cells and extrathymic T cells were abundant in the liver, but these subsets were few in the spleen. The distribution patterns of these lymphocyte subsets were unchanged in the liver and spleen, irrespective of the administration of β-glucan. In the case of the small intestine, $CD3^+$IL-$2R\beta^-$ (mainly αβT cells) and $CD3^+$IL-$2R\beta^+$ (mainly γδT cells) were present. This pattern was also unchanged by the administration of β-glucan. A similar staining pattern was also produced by two-color staining for CD3 and NK1.1 (Fig. 3A bottom). The patterns were also confirmed to be unchanged by β-glucan in this staining. $CD3^-$ $NK1.1^+$ were NK cells and $CD3^{int}NK1.1^+$ were NKT cells (approximately 50% of extrathymic T cells were NKT cells).

To further characterize the phenotype of lymphocyte subsets, stainings with various combinations were conducted, especially in the small intestine (Fig. 3B). Among IEL in the small intestine (s-IEL), $B220^+$T cells were present. Their level became low by the administration of β-glucan (35.1% → 24.9%). Two-color staining for CD4 and CD8 showed that the proportion of $CD8^+$ cells became high by β-glucan. This population was found to contain high proportions of $TCR\alpha\beta^+$ cells and $CD8\alpha^+CD8\beta^-$ cells (*i.e.*, CD8 αα homodimer cells).

We then examined the distribution of $TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ cells among $CD8\alpha^+\beta^+$ cells and $CD8\alpha^+\beta^-$ cells in the small intestine (Fig. 4). Three-color staining for CD8α, CD8β and TCRαβ (or TCRγδ) was conducted. By gated analysis, the proportion of $TCR\alpha\beta^+$ cells and $TCR\gamma\delta^+$ cells was estimated. In normal mice, approximately 60% of the CD8αβ cells were $TCR\alpha\beta^+$ and 40% of them were $TCR\gamma\delta^+$. On the other hand, approximately 30% of the CD8αα cells were $TCR\alpha\beta^+$ and 70% of them were $TCR\gamma\delta^+$. The administration of β-glucan changed this distribution pattern, namely, the proportion of $TCR\alpha\beta^+$ cells increased and that of $TCR\gamma\delta^+$ cells decreased among both CD8αβ cells and CD8αα cells.

All these experiments were repeated ($n = 4$) and the absolute numbers of various lymphocyte subsets were calculated (Fig. 5). The number of whole $CD8^+$ cells increased prominently by the administration of β-glucan ($p < 0.05$). Among $CD8^+$ cells, the increase in the number of CD8αα cells was much more prominent than that of CD8αβ cells. The number of $TCR\alpha\beta^+$ cells increased ($p < 0.05$), but that of $TCR\gamma\delta^+$ cells increased only slightly.

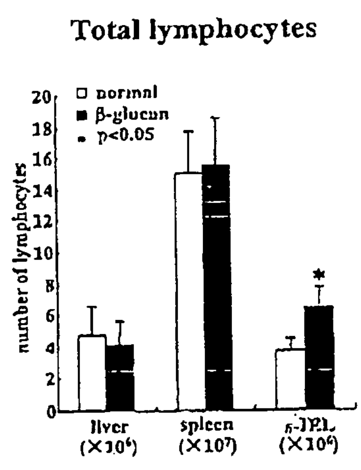

Fig. 2 Number of total lymphocytes yielded by the liver, spleen and small intestine in mice fed with or without β-glucan. Four mice were used to produce the mean and one SD.

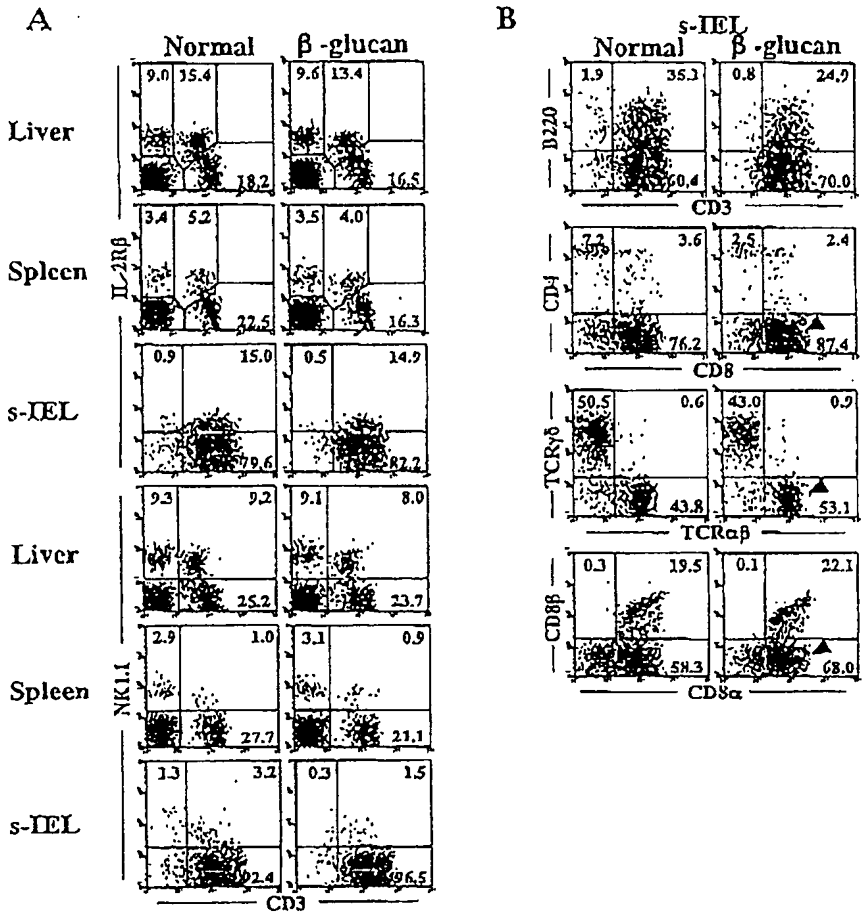

Fig. 3 Phenotypic characterization of lymphocytes by two-color immunofluorescence tests. A, Two-color staining for CD3 and IL-2Rβ (or NK1.1). B. Two-color staining for various combinations. Numbers in the figure represent the percentages of fluorescence-positive cells in corresponding areas. Representative results of three experiments are depicted.

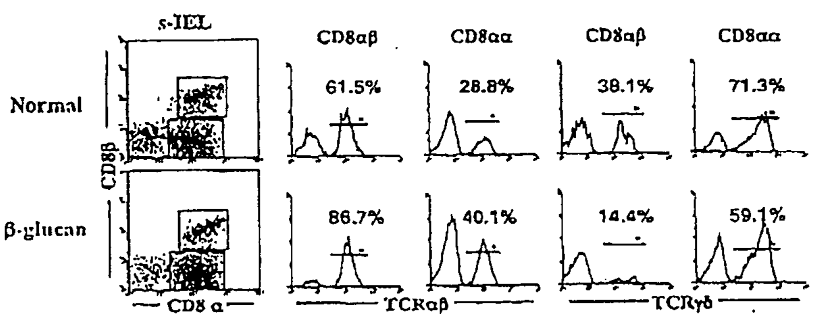

Fig. 4 Phenotypic characterization of lymphocyte subsets in the small intestine. IEL were isolated from the small intestine in mice fed with or without β-glucan. Three-color staining for CD8α, CD8β and TCRαβ (or TCRγδ) was conducted. By gated analysis, the expression of TCRαβ and TCRγδ was estimated in CD8αβ cells and CD8αα cells. Numbers in the figure represent the percentage of fluorescence-positive cells in corresponding areas.

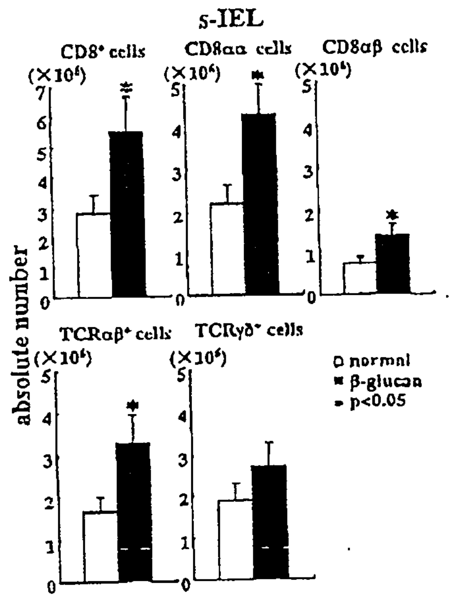

Fig. 5 Comparisons of the absolute number of lymphocyte subsets in the small intestine between mice fed without β-glucan and with β-glucan. Four mice were used to produce the mean and one SD.

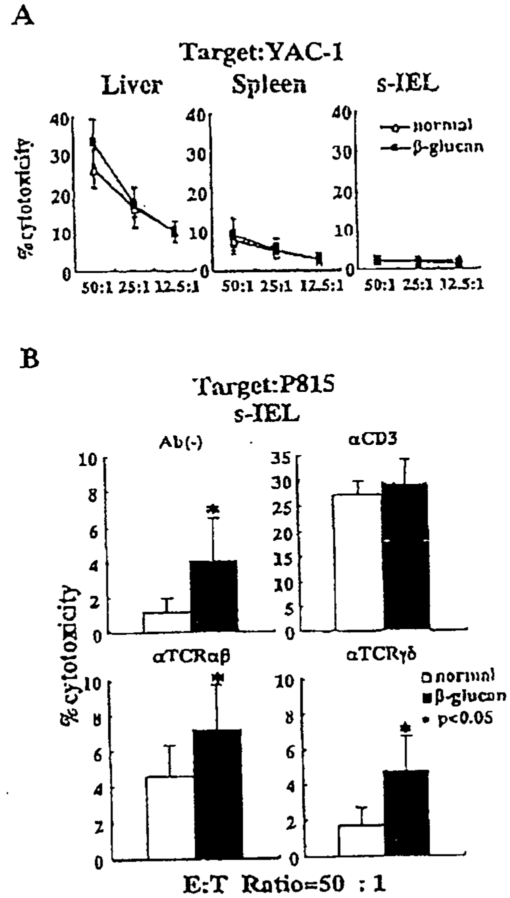

Fig. 6 Cytotoxic activity of lymphocytes. A. NK cytotoxicity against YAC-1 cells, B. Tumor cytotoxicity against P815 cells. Lymphocytes were isolated from the liver, spleen and small intestine in mice fed with or without β-glucan. Cytotoxicity was examined at the indicated effector to target (E:T) ratios. By three independent experiments, the mean and one SD were produced.

Augmented cytotoxicity of IEL in the small intestine by β-glucan

Cytotoxicity assays against YAC-1 cells (*i.e.*, NK cytotoxicity) and against P815 cells (*i.e.*, tumor cytotoxicity) were conducted (Fig. 6). Primarily, lymphocytes isolated from the liver had high NK cytotoxicity but those isolated from the spleen and small intestine had low NK cytotoxicity (9). This tendency was confirmed in mice fed with or without β-glucan (Fig. 6A). In other words, the administration of β-glucan did not have a significant effect on NK cytotoxicity.

It is known that IEL in the small intestine carry tumor cytotoxicity against P815 myeloma cells and this cytotoxicity is augmented by anti-CD3, anti-TCRαβ and anti-TCRγδ mAbs coated on assay plates (5). These experiments were conducted in IEL in the small intestine of mice fed with or without β-glucan (Fig. 6B). Under all tested conditions except of anti-CD3 mAb, IEL isolated from mice fed with β-glucan showed augmented levels of tumor cytotoxicity ($p < 0.05$).

Cytokine levels increased in sera by administration of β-glucan

As is well-known, pro-inflammatory cytokines such as TNF-α are important for host defense. Furthermore, several investigators have reported that the production of cytokines (*e.g.* TNFα and IL-1) in mice was enhanced by administration of β-glucan (11, 12). To determine if cytokine levels increased by the administration of β-glucan, in a final portion of these experiments, the levels of TNFα and IL-2 were examined in sera of mice fed with or without β-glucan (Fig. 7). In the sera of mice that underwent the administration of β-glucan, CBA assay detected prominently increased levels of TNFα (3.2 pg/mL → 5.4 pg/mL) and IL-2 (1.6 pg/mL → 3.6 pg/mL),

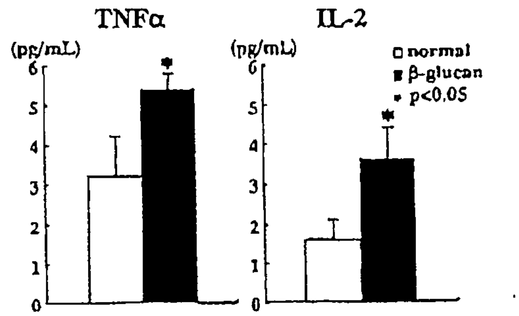

Fig. 7 Cytokine production from sera. Lymphocytes were isolated from mice fed with or without β-glucan. By three experiments, the mean and one SD were produced.

compared to levels in the mice without β-glucan ($n = 3$, $p < 0.05$). The levels of IFN-γ, IL-4 and IL-5 were also examined, but were unchanged by the oral administration of β-glucan (data not shown).

DISCUSSION

In the present study, we demonstrated that micellary β-1,3-glucan prepared from shiitake extracts was able to potentiate intestinal immunity. The number of lymphocytes yielded by the small intestine increased and the tumor cytotoxicity against P815 cells was augmented by the oral administration of micellary β-glucan. Although there have been some reports that crude-sized β-glucan potentiates intestinal immunity (19), we herein used superfine particles of β-glucan dispersed with lecithin micelles. The smaller amounts of micellary β-glucan seemed to be more effective for the potentiation of immunity than crude-sized β-glucan. The earlier reports showed that crude-sized β-glucan was required at the level of 10–30 mg/day/mouse for immunopotentiation (19). However, less than 1 mg/day/mouse of micellary β-glucan was effective for the same level of potentiation.

Primarily, β-1,3-glucan is the major component of oats, mushrooms and yeasts (2, 6, 10, 14, 17, 21). Since β-1,3-glucan is an indigestive sugar for humans, almost all of the large fragments of this sugar might be unabsorbed from the small intestine. It is speculated that this situation explains the limited function of crude-sized β-1,3-glucan for the immunopotentiation of intestinal immunity (3, 4, 15). To overcome this situation, we prepared micellary mushroom extracts containing superfine particles of β-1,3-glucan, using lecithin micelles and a high-pressure emulsifier. The crude-sized β-glucan ranged from 10 to 1000 μm of diameter, whereas micellary β-glucan ranged from 0.05 to 0.2 μm in diameter. In other words, findings of the present study suggest that a small amount of micellary β-glucan is sufficient to potentiate intestinal immunity. Some patients such as those with digestive tract malignancy might have difficulty in taking a large amount of β-glucan. In that case, micellary β-glucan might be very useful.

Similar to other data on crude-sized β-glucan (19), an increase in the number of lymphocytes in the small intestine was prominent even by the oral administration of micellary β-glucan. However, relative enrichment of $CD8\alpha\beta^+TCR\alpha\beta^+$ cells was unique. On the other hand, the proportion of $CD8\alpha\alpha^+TCR\gamma\delta^+$ cells slightly declined (see Fig. 4). These results suggest that phylogenetically developed T cells in the intestine are efficiently activated by micellary β-glucan. These results are also related to the augmentation of tumor cytotoxicity and the increased production of TNF-α and IL-2. Our preliminary experiments revealed that $CD8\alpha\beta^+TCR\alpha\beta^+$ cells had a higher ability of the above-mentioned functions (data not shown). In sharp contrast, $CD8\alpha\alpha^+$ (CD8 homodimer) cells and $TCR\gamma\delta^+$ cells are known to be the most primitive form of lymphocyte subsets in phylogeny. In any case, micellary β-glucan may be expected to be effective for the potentiation of intestinal immunity in other animals and humans.

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